(12) **EUROPEAN PATENT APPLICATION**

(54) **Solid-phase hybridization assay using anti-hybrid antibodies.**

(57) A nucleic acid hybridization method for detecting a particular polynucleotide sequence present in a virus or cell contained in a test sample such as a biological fluid. The test sample is treated to release nucleic acids from the virus or cell of interest in single stranded form and to immobilize such nucleic acids on a solid substrate such as a filter membrane. Release of nucleic acids can be accomplished with alkali, lytic enzymes, detergents, and the like. The immobilized nucleic acids are contacted with a probe to form DNA·RNA or RNA·RNA hybrids which are then detectable by binding of an anti-hybrid antibody reagent, preferably labeled. The method does not require the use of labeled probe and provides quantitative results due to the efficiency and stability of the immobilization of sample nucleic acids.

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SOLID-PHASE HYBRIDIZATION ASSAY
USING ANTI-HYBRID ANTIBODIES

BACKGROUND OF THE INVENTION

5   This invention relates to nucleic acid
hybridization assay methods for detecting specific
polynucleotide sequences. Such methods find
application in the fields of human and veterinary
medicine, agriculture, and food science, among
others. In particular, the technique can be used
10   to detect and identify etiological agents such as
bacteria and viruses, to screen bacteria for
antibiotic resistance, and to detect cancerous
cells.

The state-of-the-art nucleic acid
15   hybridization assay techniques involve chemical
modification of either the probe nucleic acid or
sample nucleic acids for the purpose of labeling
and detection. The necessity of chemically
modifying nucleic acids severely limits the
20   practical use of the technique since it requires
the large-scale preparation of labeled probes
involving complicated and expensive synthetic and
purification procedures or the *in situ* synthesis of
labeled sample nucleic acids by the analyst. In
25   particular, the resulting labeled polynucleotide
must retain the ability to hybridize efficiently
with its complementary sample or probe

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sequence. Such a requirement severaly limits the availability of useful synthetic approaches to label modification of polynucleotides intended for use in hybridization assays.

5   The early hybridization techniques involved the use of radioactive labels such as ^{3}H, ^{32}P, and ^{125}I. Labeled probes are synthesized enzymatically from radiolabeled nucleotides and a polynucleotide by such techniques as nick translation, end

10   labeling, second strand synthesis, reverse transcription, and transcription. Thus, an additional requirement of such enzymatic methods is that the modified or labeled nucleotides must serve as effective substrates for the polymerase enzymes

15   involved in the assembly of the labeled polynucleotide. Direct chemical modification of the polynucleotide is also possible, however, such methods are generally quite inefficient in incorporating labels into the polynucleotide and

20   can affect the ability of the polynucleotide to undergo hybridization.

    Because of the handling and storage disadvantages of radiolabeled materials, there has been considerable continuing efforts to develop

25   useful nonradioisotopic labeling approaches. Such labels have included light emitting molecules such as fluorescers and chemiluminescers, and ligand molecules which are capable of being specifically bound by counterpart binders which are in turn

30   labeled with detectable chemical groups such as fluorescers and enzymes. Examples of ligand labels are haptens, which are specifically bound by antibodies, and other small molecules for which

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specific binding proteins exist, e.g., biotin which
is bound by avidin.

British Pat. No. 2,019,408 describes
polynucleotide probes which are labeled with biotin
through cytochrome C linking groups and which are
then detectable by enzyme-labeled avidin. An
alternative approach to labeling probes with low
molecular weight ligands such as biotin is
described in European Pat. Appln. 63,879. In this
technique, a 5-allylamine-deoxyuridine triphosphate
(dUTP) derivative is condensed with the desired
ligand label and the thus modified nucleotide is
incorporated by standard enzymatic methods into the
desired probe.

Techniques for detecting directly the
polynucleotide duplex formed as the product of
hybridization between the sample and probe
polynucleotides, and thereby dispensing with the
chemical labeling of one or the other
polynucleotide, have been attempted. Antibodies
which will selectively bind double stranded DNA·DNA
hybrids over single stranded DNA have not been
successfully produced [Parker and Halloran,
"Nucleic Acids in Immunology", ed. Plescia and
Braun, Springer-Verlag, NY (1969) pp. 18 et seq].
Some success has been achieved in generating
antibodies that will bind DNA·RNA mixed hybrids and
have low affinity for the single stranded
polynucleotides [Rudkin and Stollar, Nature
265:472(1977); Stuart et al,
PNAS(USA)78:3751(1981); Reddy and Sofer, Biochem.
Biophys. Res. Commun. 103:959(1981); and Nakazato,
Biochem. 19:2835(1980)].

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The methods described in the literature involving the use of antibodies to DNA·RNA hybrids have all been restricted to a technique commonly referred to as *in situ* hybridization. In such technique, whole cells or virus particles from a test sample, usually a tissue section, are fixed onto microscope slides and the DNA in the nucleus is exposed by treatment with appropriate reagents such as ethanol, acetic acid, and enzymes. The exposed DNA is then hybridized with an RNA probe and the hybrids detected by fluorescence microscopy using fluorescently-labeled antibody to DNA·RNA. The cellular or viral DNA becomes in a sense immobilized on the microscope slide by being noncovalently adsorbed to the various cellular or viral elements left fixed to the slide. It is well-accepted in the art that the *in situ* hybridization technique is time consuming and unreliable and is only useful when the assay can be performed upon single cells (U.S. Pat. No. 4,483,920). Because of the variability and inefficiency in the immobilization and exposure of the sample DNA from the cell or virus inherent in such a process, its use has been restricted to qualitative analysis.

In other typical hybridization assays, a small amount of the labeled probe binds nonspecifically to the solid support used to immobilize the sample nucleic acids. This non-specifically bound probe gives a background signal which often sets the lower limit for detection of hybridized probe. The usual way to maintain the background signal at an acceptable level is to keep the probe concentration low during the hybridization step. However, this

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tactic also slows the hybridization rate and
extends the assay time.

Accordingly, there is a need to develop
hybridization techniques which do not require
labeling of the probe or sample nucleic acids and
which provide an efficient and reproducible
immobilization step. Further, such techniques
should allow the use of a variety of labels,
particularly of the nonradioisotopic type. Also,
there is a need for techniques which permit the use
of large excesses of the probe for faster
hybridization kinetics. These and other features
are objects of the present invention.

The use of antibodies to DNA·RNA or RNA·RNA
hybrids in other types of nucleic acid
hybridization assays is described in
European Patent Application No. 146 039
entitled "Hybridization Assay with Immobilization
of Hybrids by Anti-Hybrid Binding" and European
Patent Application No. 163 220 entitled "Nucleic
Acid Hybridization Assay Employing Detectable
Anti-hybrid Antibodies"

SUMMARY OF THE INVENTION

A nucleic acid hybridization method is
provided by the present invention for determining
particular polynucleotide sequences in a virus or
cell contained in a test sample, usually a
biological fluid. The test sample is treated to
release a significant detectable amount of nucleic
acid from the cell or virus of interest in single
stranded form and to immobilize such single

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stranded sample nucleic acids on a solid substrate.
The immobilized nucleic acids are contacted with a
soluble polynucleotide probe having a substantially
complementary base sequence to the sequence to be

5   detected. When the sequence to be detected is RNA,
the probe is substantially composed of DNA or RNA,
but when such sequence is DNA, the probe will be
substantially composed of RNA. Hybridization
between the probe and sequence of interest is

10   allowed to proceed under favorable conditions. The
resulting DNA·RNA or RNA·RNA hybrids are detected
by adding an antibody reagent selective for binding
such hybrids and then determining the antibody
reagent that becomes bound to such hybrids by

15   conventional methods, such as the use of a labeled
form of the antibody reagent.

        A principal feature of the present method is
that the sample nucleic acids are released and
separated from the major cellular or viral elements

20   in the sample. In the process, the sample nucleic
acids of interest if present as double stranded DNA
will be denatured to single stranded form. The
single stranded nucleic acids are then immobilized
directly onto a suitable solid substrate. The

25   overall immobilization process is thus independent
of fixation of the sample nucleic acid to cellular
or viral structural elements as is seen in the
prior art *in situ* techniques. By appropriate
selection of immobilizing substrates, an efficient

30   and stable assay element can be achieved, enabling
reproducible and quantitative analyses to be
carried out.

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DESCRIPTION OF THE PREFERRED EMBODIMENTS

Sample Treatment

A variety of materials and steps can be used
in accomplishing the immobilization step of the

5 present invention. One can first treat the test
sample, whether a biological fluid or tissue
sample, to produce an aqueous solution or
suspension of nucleic acids released from the cells
or viruses in the sample, and then, with or without

10 first separating out the resulting cellular or
viral debris, contact the solution or suspension
with the desired immobilization substrate.
Alternatively, one can collect or fix the sample
onto a suitable substrate and then treating the

15 deposited cells or viruses to release their nucleic
acids for immobilization directly on the substrate,
followed preferably by a wash to remove cellular or
viral debris.

The release of a significant detectable amount

20 of the nucleic acids in viruses or cells in the
test sample can be accomplished in several ways.
Normally, this involves treatment with alkali,
lytic enzymes, and/or detergents useful for this
purpose. Alkaline conditions such as 0.1 to 1.0

25 molar sodium hydroxide have been used to release
DNA from bacteria [see Mosely et al (1982) J. Inf.
Dis. 145:863] and this method also denatures the
DNA for immobilization and hybridization. Other
methods expose the bacteria to lytic enzymes such

30 as lysozyme or lysostaphin followed by a detergent

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such as sodium dodecylsulfate or Triton. Some
bacteria can be lysed with detergent alone.

Blood cells, such as lymphocytes can be lysed
by exposure to detergents such as sodium
5   dodecylsulfate. In the case of tissue samples, it
may be desirable to digest with a protease such as
proteinase K or a protease in combination with a
detergent and sodium perchlorate [Lizardi and
Engelberg (1979) Anal. Biochem. 98:116]. In
10 addition, a cell lysate (digest) can be extracted
with phenol-chloroform, particularly if the cell
debris interfere with the immobilization of the
nucleic acids. Bresser et al (1983) DNA 2:243
describes a method for treating cell lysates with
15 hot sodium iodide to solubilize nucleic acids and
to facilitate immobilization on nitrocellulose.

Viral nucleic acids can usually be dissociated
from coat proteins by exposure to detergents such
as sodium dodecylsulfate [Virtanen et al (1983)
20 Lancet 381; Teramato et al (1984) J. Clin. Micro
20:373]. Where desirable, digestion with a
proteolytic enzyme and extraction with phenol can
also be employed [Wichendom et al (1985) Lancet
65]. The nucleic acids are usually denatured with
25 alkali or high temperature to facilitate
immobilization and hybridization. High temperature
is preferable for denaturation of double stranded
RNA because it is destroyed by alkali.

Immobilization

30 As mentioned above, the immobilization step
can actually be accomplished simultaneously with or
subsequent to the release, and optional

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denaturation, treatment. Essentially any available means can be used to immobilize the single stranded sample nucleic acids. Normally one will select from adsorption methods, covalent methods, and dual hybridization protocols.

The solid substrate can take on a variety of shapes and compositions, including microparticles, beads, porous and impermeable strips and membranes, the interior surface of reaction vessels such as test tubes and microtiter plates, and the like. Means for attaching the sample nucleic acids to a selected solid support will be a matter of routine skill to the worker in the field.

One method for adsorbing the sample nucleic acids onto nitrocellulose membranes involves saturating a solution of the nucleic acids with sodium iodide and spotting br filtering aliquots onto the membrane [Bresser et al (1983) DNA 2:243]. The sodium iodide facilitates denaturation of the nucleic acids and enhances adsorption onto the membrane. Alternatively, the nucleic acids can be treated with glyoxal, usually at concentrations around 1 molar, and then adsorbed onto the membrane. This method is particularly advantageous for RNA. The nucleic acids are fixed by baking at around 80°C under vacuum for a period in the range of 2-4 hours. [Thomas, P.S., (1983) Meth. in Enzymol. 100:255].

Covalent immobilization of nucleic acids can also be accomplished. A wide variety of support materials and coupling techniques can be employed. For example, the nucleic acids can be coupled to phosphocellulose through phosphate groups activated by carbodiimide or carbonyldiimidazole [Bautz,

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E.K.F., and Hall, B.D., (1962) Proc. Nat'l. Acad. Sci. USA 48:400-408; Shih, T.Y., and Martin, M.A., (1974) Biochem. 13:3411-3418]. Also, diazo groups on m-diazobenzoyloxymethyl cellulose can react with guanine and thymidine residues of the polynucleotide [Noyes, B.E., and Stark, G.R., (1975) Cell 5:301-310; Reiser, J., et al, (1978) Biochem. Biophys, Res. Commun. 85:1104-1112]. Polysaccharide supports can also be used with coupling through phosphodiester links formed between the terminal phosphate of the polynucleotide and the support hydroxyls by water soluble carbodiimide activation [Richwood, D., (1972) Biochim. Biophys. Acta 269:47-50; Gilham, P.T., (1968) Biochem. 7:2809-2813], or by coupling nucleophilic sites on the polynucleotide with a cyanogen bromide activated support [Arndt-Jovin, D.J., et al, (1965) Eur. J. Biochem. 54:411-418; Linberg, U., and Eriksson, S., (1971) Eur. J. Biochem. 18:474-479]. Further, the 3'-hydroxyl terminus of RNA can be oxidized with periodate and coupled by Schiff base formation with supports bearing amine or hydrazide groups [Gilham, P.T., (1971) Method, Enzymol. 21:191-197; Hansske, H.D., et al, (1979) Method. Enzymol. 59:172-181]. Supports having nucleophilic sites can be reacted with cyanuric chloride and then with the polynucleotide [Hunger, H.D., et al, (1981) Biochim. Biophys. Acta 653:344-349].

Another approach to immobilizing the released single stranded sample nucleic acids involves the dual hybridization procedure as known in the art [Methods in Enzymol. 65:468(1968) and

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Gene 21:77-86(1983)]. Following such a method, a
second probe is used which has a base sequence
complementary to a mutually exclusive portion of
the sequence of interest relative to the first
probe. The second probe is immobilized on the
5   solid substrate using any of the previously
described techniques or any other useful technique,
and by hybridization with the sequence to be
detected, thereby immobilizes same for detection by
hybridization with the first probe.
10   A specialized modification of the dual
hybridization method can be applied to measurements
of messenger RNAs (mRNA) from eukaryotic cells
which have poly(rA) tails on the 3'-OH ends.
Polyadenylated RNAs in cell lysates can be
15   immobilized by hybridization to immobilized
poly(rT) or poly(rU). Then a specific DNA probe
can be hybridized with the immobilized mRNAs and if
a complementary sequence is present the DNA·RNA
hybrid can be detected with labeled antibody to
20   DNA·RNA.

The Probe

          The probe will comprise at least one single
stranded base sequence substantially complementary
to the sequence to be detected. However, such base
25   sequence need not be a single continuous
polynucleotide segment, but can be comprised of two
or more individual segments interrupted by
noncomplementary sequences. These nonhybridizable
sequences can be linear, or they can be
30   self-complementary and form hairpin loops. In
addition, the complementary region of the probe can

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be flanked at the 3'- and 5'-termini by
nonhybridizable sequences, such as those comprising
the DNA or RNA of a vector into which the
complementary sequence had been inserted for
5   propagation. In either instance, the probe as
presented as an analytical reagent will exhibit
detectable hybridization at one or more points with
sample nucleic acids of interest. Linear or
circular single stranded polynucleotides can be
10   used as the probe element, with major or minor
portions being duplexed with a complementary
polynucleotide strand or strands, provided that the
critical homologous segment or segments are in
single stranded form and available for
15   hybridization with sample DNA or RNA, and provided
that the antibody reagent selected for use with the
probe does not significantly crossreact with the
double stranded regions in the probe (e.g., where
the antibody reagent is specific for DNA·RNA
20   hybrids and the probe comprises RNA·RNA double
stranded regions, or vice versa). The
complementary probe sequence can be of any
convenient or desired length, ranging from as few
as a dozen to as many as 10,000 bases, and
25   ·including oligonucleotides having less than about
50 bases.

    The RNA or DNA probe can be obtained in a
variety of conventional manners. For example, in
the case of RNA probes, RNA can be isolated as the
30   natural products of cells, such as 5s, 16s and 23s
ribosomal RNAs from bacteria or cellular transfer
RNAs. It is also practial to isolate specific
messenger RNAs from cells which specialize in

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production of large amounts of a protein for which
the messenger codes.

5   In vitro synthesis of RNA probes can be
accomplished with a vector which contains the very
active Salmonella typhimurium bacteriophage SP6
transcription promoter [Green et al (1983) Cell
32:681]. A vector with multiple restriction
endonuclease sites adjacent to the promoter is
available from Promega Biotec, Madison, WI, USA. A
10   DNA probe is cloned into the vector which is then
propagated in a bacterial host. Multiple RNA
copies of the cloned DNA probe can be synthesized
in vitro using DNA dependent RNA polymerase from
bacteriophage SP6.

15   DNA probes can be prepared from a variety of
sources. An entire bacterial genome can be
immobilized for a hybridization assay designed to
detect bacteria in a typically sterile sample. The
assay would be capable of detecting abundant
20   bacterial RNAs such as ribosimal RNAs and transfer
RNAs. Alternatively, specific DNA sequences
complementary to cellular RNAs can be cloned into
well known plasmid or viral vectors and used as
hybridization probes.

25   It should be understood that in using the
expressions "RNA probe" and "DNA probe" herein, it
is not implied that all nucleotides comprised in
the probe be ribonucleotides or
2'-deoxyribonucleotides. The fundamental feature
30   of an RNA or DNA probe for purposes of the present
invention is that it be of such character to enable
the stimulation of antibodies to DNA·RNA or RNA·RNA
hybrids comprising an RNA or DNA probe which do not
crossreact to an analytically significant degree

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with the individual single strands forming such
hybrids. Therefore, one or more of the
2'-positions on the nucleotides comprised in the
probe can be chemically modified provided the
5   antibody binding characteristics necessary for
performance of the present assay are maintained to
a substantial degree. Likewise, in addition or
alternatively to such limited 2'-deoxy
modification, a probe can have in general any other
10   modification along its ribose phosphate backbone
provided there is no substantial interference with
the specificity of the antibody to the double
stranded hybridization product compared to its
individual single strands.

15   Where such modifications exist in an RNA or
DNA probe, the immunogen used to raise the antibody
reagent would preferably comprise one strand having
substantially corresponding modifications and the
other strand being substantially unmodified RNA or
20   DNA, depending on whether sample RNA or DNA was
intended to be detected. Preferably, the modified
strand in the immunogen would be identical to the
modified strand in an RNA or DNA probe. An example
of an immunogen is the hybrid
25   poly(2'-0-methyladenylic
acid)·poly(2'-deoxythymidylic acid). Another would
be poly(2'-0-ethylinosinic acid)·poly(ribocytidylic
acid). The following are further examples of
modified nucleotides which could be comprised in a
30   modified probe: 2'-0-methylribonucleotide,
2'-0-ethylribonucleotide,
2'-azidodeoxyribonucleotide,
2'-chlorodeoxyribonucleotide,
2'-0-acetylribonucleotide, and the

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phosphorothiolates or methylphosphonates of
ribonucleotides or deoxyribonucleotides. Modified
nucleotides can appear in probes as a result of
introduction during enzymic synthesis of the probe

5   from a template. For example, adenosine
5'-0-(1-thiotriphosphate) (ATPαS) and dATPαS are
substrates for DNA dependent RNA polymerases and
DNA polymerases, respectively. Alternatively, the
chemical modification can be introduced after the

10   probe has been prepared. For example, an RNA probe
can be 2'-0-acetylated with acetic anhydride under
mild conditions in an aqueous solvent [Steward, D.
L. et al, (1972) Biochim. Biophys. Acta 262:227].

       The critical property of an RNA or DNA probe

15   for use herein is that antibodies raised against
the probe duplexed with a complementary RNA or DNA
strand, as desired, will discriminate in their
binding properties between the duplexed form of the
probe and single stranded nucleic acids. It is

20   this property which enables detection of hybridized
probe in the assay mixture without significant
background binding to the unhybridized single
stranded form of the probe or any nonspecifically
bound single stranded sample nucleic acids. While

25   as described above certain modifications along the
ribonucleotide or deoxyribonucleotide strand can be
tolerated without loss of antibody discrimination
of the duplex from single strands, it will
generally be preferable to employ RNA probes which

30   are composed entirely of ribonucleotides when the
sample polynucleotide is RNA or DNA. DNA probes
can be used advantageously when the sample is RNA.

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Anti-Hybrid Antibody Reagent and Detection Schemes

The antibody reagent of the present invention
is principally characterized by its ability to bind
the DNA·RNA or RNA·RNA hybrids formed between the
probe and complementary sample nucleic acids to the
5   significant exclusion of single stranded
polynucleotides. The antibody reagent can consist
of whole antibodies, antibody fragments,
polyfunctional antibody aggregates, or in general
10   any substance comprising one or more specific
binding sites from an antibody for RNA·RNA or
DNA·RNA, as the case may be. When in the form of
whole antibody, it can belong to any of the classes
and subclasses of known immunoglobulins, e.g., IgG,
15   IgM, and so forth. Any fragment of any such
antibody which retains specific binding affinity
for the hybridized probe can also be employed, for
instance, the fragments of IgG conventionally known
as Fab, F(ab'), and F(ab')$_2$. In addition,
20   aggregates, polymers, derivatives and conjugates of
immunoglobulins or their fragments can be used
where appropriate.
The immunoglobulin source for the antibody
reagent can be obtained in any available manner
25   such as conventional antiserum and monoclonal
techniques. Antiserum can be obtained by
well-established techniques involving immunization
of an animal, such as a mouse, rabbit, guinea pig
or goat, with an appropriate immunogen. The
30   immunoglobulins can also be obtained by somatic
cell hybridization techniques, such resulting in
what are commonly referred to as monoclonal

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antibodies, also involving the use of an appropriate immunogen.

Immunogens for stimulating antibodies specific for DNA·RNA hybrids can comprise homopolymeric or heteropolymeric polynucleotide duplexes. Among the possible homopolymer duplexes, particularly preferred is poly(rA)·poly(dT) [Kitagawa and Stollar (1982) Mol. Immunol. 19:413]. However, in general, heteropolymer duplexes will be preferably used and can be prepared in a variety of ways, including transcription of ϕX174 virion DNA with RNA polymerase [Nakazato (1980) Biochem. 19:2835]. The selected RNA·DNA duplexes are adsorbed to a methylated protein, or otherwise linked to a conventional immunogenic carrier material, such as bovine serum albumin, and injected into the desired host animal [see also Stollar (1980) Meth. Enzymol. 70:70].

Antibodies to RNA·RNA duplexes can be raised against double stranded RNAs from viruses such as reovirus or Fiji disease virus which infects sugar cane, among others. Also, homopolymer duplexes such as poly(rI)·poly(rC) or poly(rA)·poly(rU), among others, can be used for immunization as above.

The binding of the antibody reagent to the hybridized probe duplex according to the present method can be detected by any convenient technique. Advantageously, the antibody reagent will itself be labeled with a detectable chemical group. Such detectable chemical group can be any material having a detectable physical or chemical property. Such materials have been well-developed in the field of immunoassays and in general most any label

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useful in such methods can be applied to the
present invention. Particularly useful are
enzymatically active groups, such as enzymes (see
Clin. Chem. (1976)22:1243, U.S. Reissue Pat. No.

5   31,006 and UK Pat. 1,019,408), enzyme substrates
(see U.S. Pat. No. 4,492,751, cofactors (see U.S.
Pat. Nos. 4,230,797 and 4,238,565), and enzyme
inhibitors (see U.S. Pat. No. 4,134,792);
fluorescers (see Clin. Chem. (1979)25:353);

10   chromophores; luminescers such as chemiluminescers
and bioluminescers (see U.S. Pat. No. 4,380,580);
specifically bindable ligands such as biotin (see
European Pat. Spec. 63,879) or a hapten (see PCT
Publ. 83-2286); and radioisotopes such as ^{3}H, ^{35}S,

15   ^{32}P, ^{125}I, and ^{14}C. Such labels and labeling pairs
are detected on the basis of their own physical
properties (e.g., fluorescers, chromophores and
radioisotopes) or their reactive or binding
properties (e.g., enzymes, substrates, cofactors

20   and inhibitors). For example, a cofactor-labeled
antibody can be detected by adding the enzyme for
which the label is a cofactor and a substrate for
the enzyme. A hapten or ligand (e.g., biotin)
labeled antibody can be detected by adding an

25   antibody to the hapten or a protein (e.g., avidin)
which binds the ligand, tagged with a detectable
molecule. Such detectable molecule can be some
molecule with a measurable physical property (e.g.,
fluorescence or absorbance) or a participant in an

30   enzyme reaction (e.g., see above list). For
example, one can use an enzyme which acts upon a
substrate to generate a product with a measurable
physical property. Examples of the latter include,
but are not limited to, β-galactosidase, alkaline

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phosphatase and peroxidase. Other labeling schemes will be evident to one of ordinary skill in the art.

Alternatively, the antibody reagent can be detected based on a native property such as its own antigenicity. A labeled anti-(antibody) antibody will bind to the primary antibody reagent where the label for the second antibody is a conventional label as above. Further, antibody can be detected by complement fixation or the use of labeled protein A, as well as other techniques known in the art for detecting antibodies.

Where the antibody reagent is labeled, as is preferred, the labeling moiety and the antibody reagent are associated or linked to one another by direct chemical linkage such as involving covalent bonds, or by indirect linkage such as by incorporation of the label in a microcapsule or liposome which is in turn linked to the antibody. Labeling techniques are well-known in the art and any convenient method can be used in the present invention.

Reaction Mixture

The test sample to be assayed can be any medium of interest, and will usually be a liquid sample of medical, veterinary, environmental, nutritional, or industrial significance. Human and animal specimens and body fluids particularly can be assayed by the present method, including urine, blood (serum or plasma), milk, cerebrospinal fluid, sputum, fecal matter, lung aspirates, throat swabs,

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genital swabs and exudates, rectal swabs, and
nasopharnygal aspirates.

As is known in the art, various hybridization
conditions can be employed in the assay.
Typically, hybridization will proceed at slightly
elevated temperatures, e.g., between about 35 and
75°C and usually around 65°C, in a solution
comprising buffer at pH between about 6 and 8 and
with appropriate ionic strength (e.g., 2XSSC where
1XSSC = 0.15M sodium chloride and 0.015M sodium
citrate, pH 7.0), protein such as bovine serum
albumin, Ficoll (a trademark identifying a
copolymer of sucrose and epichlorohydrin sold by
Pharmacia Fine Chemicals, Piscataway, NJ, USA),
polyvinylpyrrolidone, and a denatured foreign DNA
such as from calf thymus or salmon sperm. The
degree of complementarity between the sample and
probe strands required for hybridization to occur
depends on the stringency of the conditions as
known in the art.

Normally, the temperature conditions selected
for hybridization will be incompatible with the
binding of antibody reagent to formed hybrids and
detection of the label response. Accordingly, the
antibody reagent binding step and label detection
step will proceed after completion of the
hybridization step. The reaction mixture will
usually be brought to a temperature in the range of
from about 3°C to about 40°C and the binding and
detection steps then performed.

It might be found in a particular assay
situation using an RNA probe that the probe is
subject to partial degradation by alkaline
hydrolysis of the phosphodiester bonds or by the

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presence of ribonucleases. In the former case, hydrolysis can be controlled by avoiding exposure of the probe to a pH higher than about 10. Ribonucleases can be effectively inhibited by the presence of such substances as sodium dodecylsulfate, aurintricarboxylic acid, ribonucleoside vanadyl complexes, heparin, diethylpyrocarbonate, and proteinaceous inhibitors isolated from mammalian sources.

The present invention will now be illustrated, but is not intended to be limited, by the following examples.

Example I

Hybridization Assay for Detection of Bacteria in Urine

A. Preparation of methylated thyroglobulin

One hundred milligrams of bovine thyroglobulin (Sigma Chemical Co., St. Louis, MO, USA) is combined with 10 ml of anhydrous methanol and 400 μl of 2.55 M HCl in methanol. This mixture is stirred on a rotary mixer at room temperature for 5 days. The precipitate is collected by centrifugation and washed twice with methanol and twice with ethanol.

B. Preparation of antibody to DNA·RNA hybrid

A DNA·RNA hybrid is prepared by transcription of øX174 virion DNA with RNA polymerase as described by Nakazato [Biochem. 19:2835(1980)].

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One hundred fifty (150) micrograms (µg) of the hybrid in 250 µL of 20 mM Tris-hydrochloride buffer, pH 7.4, 1 mM ethylenediaminetetraacetate (EDTA) is combined with 150 µg of methylated
5   thyroglobulin in 250 µL water. A precipitate forms and is suspended in Tris-buffer. This mixture is emulsified with an equal volume of Freunds adjuvant. BALB/c mice are immunized subcutaneously with 10 µg of hybrid per injection. The
10   immunizations are repeated on days 14, 21, 28 and 35 and test bleedings are taken on days 23 and 37. Serums are titered for antibodies by enzyme immunoassay using Immunolon II microtiter plates (Dynatech, Alexandria, VA) in which the wells are
15   coated with DNA·RNA hybrid. The coating is accomplished by placing 100 µL of 0.015M sodium citrate, pH 6.8, 0.15M NaCl containing 5.0 µg DNA·RNA/mL in each well and allowing it to stand for 2 hours at room temperature. Then the wells
20   are washed three times with 20 mM sodium phosphate buffer, pH 7.4, 0.15M NaCl, 5 mg bovine serum albumin/mL and 0.5% (v/v) Tween 20.

Spleens from mice with high titers to DNA·RNA and low titers to single stranded DNA are fused
25   with SP 2/0-Ag 14 myeloma cells and the resulting hybridomas are screened for those producing antibodies specific for DNA·RNA [Stuart et al (1981) Proc. Natl. Acad. Sci. USA 78, 3751, Galfre and Milstein (1981) Meth. in Enzymol. 73, 1]. A
30   particularly preferred hybridoma is that deposited with the American Type Culture Collection, Rockville, MO, USA, as ATCC HB 8730.

The cloned hybridomas are propagated in the peritoneal cavity of mice to generate a large

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quantity of antibody. The antibody is purified
from the ascites fluid by high pressure liquid
chromatography on an anion-exchange column,
Bakerbond MAb^{TM} (J. T. Baker Research Products,
Glenn Ellyn, IL, USA). The ascites fluid is
dialyzed into 10 mM potassium phosphate buffer, pH
6.8, centrifuged to remove precipitate and passed
through a 0.22 µm nitrocellulose filter. One to
two milliliters of ascites fluid is applied to a 10
x 250 mm anion-exchange column equilibrated with 10
mM potassium phosphate buffer, pH 6.84. The
chromatography is developed with a 60 min linear
gradient from 10 mM potassium phosphate buffer, pH
6.85 to 85 mM potassium phosphate, pH 6.40 at a
flow rate of 1.0 mL/min. The absorbance of the
effluent at 254 nm is monitored and the peak with
the IgG is collected.

C. Labeling of antibody to DNA·RNA with
β-Galactosidase

The purified antibody to DNA·RNA is dialyzed
against 0.1 M sodium acetate buffer, pH 4.2,
overnight and any insoluble material that forms is
removed by centrifugation. A 3.3 mL aliquot
containing 10.5 mg IgG is combined with 64 µL of
pepsin (5 mg/mL) and incubated at 37°C for 16
hours. The digestion is quenched by addition of
0.5 ml of 2.0 M Tris-base to give pH 8.0.

The digest is concentrated to about 0.5 ml on
a pressure dialysis membrane and then
chromatographed on a 1.4 x 57 cm column of
Sephacryl-200 (Pharmacia) equilibrated with 10 mM
sodium phosphate buffer, pH 7.0, 0.15 M NaCl.

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Fractions of 2.5 mL are collected and the absorbances at 280 nm are measured. The first peak eluted, fractions 17 to 20, are pooled to yield 4.2 mg of (Fab')$_2$.

5   Eight milligrams of β-galactosidase from E. Coli (Sigma Chemical Co., St. Louis, MO, USA) in 100 mM sodium phosphate buffer, pH 7.0, 0.15 M NaCl is combined with 35 μL of 0.1 M dithiothreitol and allowed to stand at 25°C for 4.0 hours. The

10   mixture is chromatographed on a 1 x 25 cm column of BioGel P-6DG (BioRad Laboratories, Richmond, CA, USA) equilibrated with the same buffer. One milliliter fractions are collected and the enzyme is recovered in the first peak with absorbance at

15   280 nm. The protein is concentrated to 2.5 ml and to yield 7.1 mg. This enzyme has 10.4 sulfhydryl groups as determined by the method of Ellman [Ellman (1959) Arch. Biochem. Biophys. 82:70].

     A 18.2 mM solution of

20   N,N'-o-phenylenedimaleimide (Aldrich Chemical Co., Milwaukee, WI, USA) is prepared in dimethylformamide and 121 μL is added to 2.1 ml of the reduced β-galactosidase (2.8 mg/mL). This mixture is allowed to react at room temperature for

25   1.0 hour and then it is chromatographed on the BioGel P-6DG column described above and the modified enzyme is recovered in the first eluted peak of material with absorbance at 280 nm.

     Four milligrams (1.45 ml) of the (Fab')$_2$

30   preparation described above is combined with 143 μL of 1.0 M sodium phosphate buffer, pH 7.0, 200 μL of 100 mM dithiothreitol and 20 μL of 100 mM EDTA. The mixture is allowed to stand at room temperature for 3 hours and then is chromatographed on a 1 x 25

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cm column of BioGel P-6DG equilibrated with 10 mM
sodium phosphate buffer, pH 7.0, 0.15 M NaCl and
1.0 mM EDTA. The first eluted peak of material
with absorbance at 280 nm is pooled and

5   concentrated to 2.5 mL. Typically, the protein has
an average of 2.5 sulfhydryl groups per Fab' as
determined by the method of Ellman, supra.

Four milliliters of the activated
β-galactosidase, 3.4 mg, is combined with 1.1 ml of

10   the Fab', 1.45 mg, and allowed to react for 22
hours at 4°C.

Insoluble material which forms is removed by
centrifugation and the supernatant is
chromatographed on a 1.4 x 44 cm column of BioGel

15   A1.5 m (BioRad Laboratories) equilibrated with 10
mM sodium phosphate buffer, pH 7.0, 0.15 M NaCl.
Two milliliter fractions are collected and the
absorbances at 280 nm are measured. Also, the
β-galactosidase activity in each fraction is

20   assayed using o-nitrophenyl-β-galactoside as
substrate. Fractions 14 to 18 typically have
strong absorbances at 280 nm and enzyme activity
and they are pooled.

D.   Preparation of ribosomal RNA probe

25   A mixture of 16 and 23s ribosomal RNAs is
isolated from E. coli by the method of Takanami
(1967) Meth. Enzymol. 12A:491.

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E.   Hybridization assay

A 0.45 µm pore size nitrocellulose membrane
(Schleicher and Schull, Keene, NH, USA) is mounted
in a multiwell Minifold apparatus (Schleicher and
5   Schuell) and 1.0 ml aliquots of urines suspected of
having bacteria are filtered through the membrane.
Then the membrane is placed on filter paper
saturated with 0.3 M NaOH for 3.0 hours at room
temperature. This alkali disrupts bacteria on the
10   membrane, degrades any RNA present and denatures
the DNA. The membrane is placed on filter paper
saturated with 0.2 M sodium phosphate buffer, pH
7.0, for 30 minutes to neutralize the alkali and
then the membrane is dried at 80°C in vacuo for 2
15   hours.
Hybridization is conducted by placing the
membrane in a plastic pouch containing 0.25 ml/cm^2
of a solution containing 4 parts by volume of
formamide and 6 parts 33 mM sodium phosphate
20   buffer, pH 7.7, 0.5 M NaCl, 3.3 mM EDTA, 0.1% (w/v)
sodium dodecylsulfate, 0.5 mg bovine serum
albumin/mL, 0.5 mg Ficoll/mL, 0.5 mg
polyvinylpyrrolidone/mL, 0.2 mg salmon sperm DNA/mL
and 100 ng of the purified E. coli ribosomal
25   RNA/mL. (Prior to use the salmon sperm DNA is
incubated at 37°C for 16 hours in 0.3 M NaOH and
then neutralized with 5M acetic acid.)
The plastic pouch is sealed and placed in a
water bath at 55°C for 17 hours. Then the membrane
30   is rinsed twice for 5 minutes each in 20 mM sodium
phosphate buffer, pH 7.4, 0.3 M NaCl, 0.2 mM EDTA
and 1.0 mg sodium dodecylsulfate/mL. Next it is

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washed for 15 minutes in 1.0 mL/cm^2 of 50 mM sodium phosphate buffer, pH 7.6, containing 5 mM MgCl$_2$, 5.0 mg BSA/mL, 0.5% (v/v) Tween 20. The β-galactosidase anti-DNA·RNA conjugate is diluted

5   into this sodium phosphate buffer solution at 200 ng/mL and spread uniformly over the membrane at 0.2 mL/cm^2. The wet membrane is allowed to stand at room temperature for 1 hour and care is taken to prevent drying.

10   Excess enzyme-antibody conjugate is washed from the membrane with three five minute washes each composed of 2.5 mL/cm^2 of the sodium phosphate buffer solution containing 0.5 M NaCl. Finally, enzyme-antibody conjugate bound to DNA·RNA hybrids

15   on the membrane is detected by applying 0.2 mL/cm^2 of 50 mM sodium phosphate buffer, pH 7.6, 5 mM MgCl$_2$ and 1.0 mM 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma). The substrate solution is allowed to

20   stand in contact with the membrane at room temperature for 1 hour with care to prevent drying. Areas of the membrane which filtered urines with more than 4 x 10^5 bacteria/mL develop blue color which is distinguishable from background. The test

25   is most sensitive for gram negative bacteria.

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Example II

Hybridization Assay for Cytomegalovirus in Urine

A. Preparation of an RNA probe for
    cytomegalovirus

5   A DNA fragment from the genome of
cytomegalovirus is cloned into a vector containing
the promoter for DNA dependent RNA polymerase from
bacteriophage SP6 that infects Salmonella
typhimurium LT2 cells. The cloned sequence is
10   transcribed by the polymerase to produce an RNA
probe.
        Cytomegalovirus DNA is digested with EcoRI
restriction endonuclease and the fragments are
cloned into the plasmid pACYC 184 [Tamashiro et àl
15   (1982) J. Virology 42:547-557]. The plasmids are
propagated in E. coli strain HB101 and the EcoRI e
fragment defined in the Tamashiro reference is used
for preparation of the probe. The purified plasmid
is digested with EcoRI restriction endonuclease and
20   the insert is isolated by agarose gel
electrophoresis [Maniatis et al (1982) "Molecular
Cloning", Cold Spring Harbor Laboratory]. The
cytomegalovirus EcoRI e fragment is cloned into the
EcoRI site of the pSP65 vector available from
25   Promega Biotec, Madison, WI, USA.
        The pSP65 vector with the EcoRI e insert is
propagated in E. coli JM103 cells at 37°C. The
cells are lysed and the cellular DNA is isolated by
phenol/chloroform extractions. The plasmid is
30   separated from the chromosomal DNA by

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centrifugation in a cesium chloride-ethidium bromide gradient [Maniatis et al, supra].

The cesium chloride and ethidium bromide are separated from the plasmid by gel filtration on Sephadex G-50 (Pharmacia Fine Chemicals) in 10 mM Tris-hydrochloride buffer, pH 7.5, containing 50 mM NaCl and 1 mM EDTA. The effluent containing DNA is collected and the DNA is precipitated with cold ethanol. The precipitate is dissolved in 10 mM Tris-hydrochloride buffer, pH 7.5, containing 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol and digested for 1 hour at 37°C with 1 unit Hind III restriction endonuclease per microgram DNA. The mixture is extracted once with phenol/chloroform and the DNA is precipitated with cold ethanol. The precipitate is dissolved in 10 mM Tris-hydrochloride buffer, pH 7.4, to give 0.5 mg DNA/mL.

This pSP65 vector with the EcoRI e insert is transcribed with SP6 DNA dependent RNA polymerase starting at the promoter and ending at the site cut by Hind III restriction endonuclease. Most of the DNA in the transcribed region is the EcoRI e insert.

The transcription reaction mixture (500 µL) has the following composition: 50 µg of the Hind III digested DNA; 40 mM Tris-hydrochloride buffer, pH 7.5; 6 mM $MgCl_2$; 2 mM spermine; 0.5 mM ATP, CTP, UTP and GTP; 10 mM dithiothreitol; 500 units RNasin (Promega Biotec) and 50 units of SP6 RNA polymerase (Promega Biotec). The reaction is allowed to proceed for 1 hour at room temperature and then an additional 50 units of RNA polymerase is added and allowed to react for one hour.

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DNA in the mixture is destroyed by digestion for 10 minutes at 37°C with 10 µg of RNase-free DNase. The reaction mixture is extracted with phenol/chloroform and chromatographed on Sephadex
5   G-50 in 10 mM Tris-hydrochloride buffer, pH 7.4, 0.1 M NaCl. The RNA is collected and precipitated with cold ethanol. The precipitate is dissolved in 50 mM sodium acetate buffer, pH 6.0, containing 1 mM EDTA.

10   B.   Hybridization assay

Cytomegalovirus is isolated from urine by centrifugation and the viral DNA is denatured and immobilized on a nitrocellulose membrane. The DNA is hybridized with the RNA probe and DNA·RNA
15   hybrids formed are detected with the β-galactosidase-anti-DNA·RNA conjugate.
Cells and cell debris are removed from urine by centrifugation in a Sorvall GLC-3 centrifuge at 3000 rpm for 5 minutes. Ten milliliters of
20   supernatant is placed in a polyallomer ultracentrifuge tube and run at 25,000 rpm in a Beckman Ti50 rotor for 75 minutes. The supernatant is removed and the pellet is dissolved in 50 µL of 0.1 M NaOH and incubated at 37°C for 2 hours.
25   Twenty microliters of 0.5 M sodium phosphate buffer, pH 6.0, containing 10 mM EDTA is added and the mixture is spotted on Gene Screen Plus Microporous membrane (New England Nuclear Research Products, Boston, MA, USA). The membrane is
30   allowed to air-dry to fix the DNA to the surface.
Hybridization is conducted as described in Example I above except that 0.1 µg of the

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cytomegalovirus RNA probe is used per mL of hybridization solution and the hybridization temperature is 40°C. Immunodetection of DNA·RNA hybrids is carried out as in Example I. Urines infected with cytomegalovirus give a blue spot on the membrane and uninfected urines give a color equivalent to the background observed on areas of the membrane not exposed to urine sediments.

The above examples provide representative embodiments of the present invention. Obviously many other modifications can be made without departing from the inventive features of the present method.

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Claims:

1. A method for determining a particular poly-
nucleotide sequence present in a virus or cell contained in
a test sample, wherein nucleic acids from the virus or cell
are contacted with a polynucleotide probe comprising at
least one single stranded base sequence which is substan-
tially complementary to the base sequence to be determined,
such contact being performed under conditions favorable to
hybridization between the sequence to be determined and the
complementary probe sequence, and wherein resulting hybrids
are detected,
       characterized in that the test sample is treated to
release a significant detectable amount of nucleic acids
from the virus or cell in single stranded form and to
immobilize such sample nucleic acids on a solid support, the
polynucleotide probe being (i) substantially composed of RNA
when the sequence to be determined is DNA, or (ii) substan-
tially composed of DNA or RNA when the sequence to be
determined is RNA, and the hybridized probe is detected by
adding an antibody reagent capable of binding to DNA·RNA or
RNA·RNA duplexes formed between the sequence to be de-
termined and the complementary probe sequence and determin-
ing the antibody reagent that becomes bound to such duplex-
es.

2. The method of Claim 1 wherein the test sample is
treated to release and denature a significant detectable
amount of nucleic acids from said virus or cell and the
resulting single stranded nucleic acids are immobilized on a
solid substrate.

3. The method of any of Claims 1 and 2 wherein the virus or cell is
immobilized on the solid substrate and then treated to
release and denature a significant detectable amount of

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nucleic acids therefrom, the resulting single stranded nucleic acids becoming immobilized on such substrate.

4. The method of any of Claims 1 to 3 wherein the test sample is a biological fluid and the substrate is a filter through which the biological fluid sample is passed to immobilize such virus or cell.

5. The method of any of Claims 1 to 4 wherein the single stranded sample nucleic acids are immobilized on the solid substrate by adsorption.

6. The method of any of Claims 1 to 5 wherein the single stranded sample nucleic acids are immobilized on the solid substrate by contact with a solid substrate to which is bound a polynucleotide having a base sequence complementary to a mutually exclusive portion of the nucleic acid containing the sequence to be determined and to which the probe hybridizes.

7. The method of any of Claims 1 to 6 wherein the antibody reagent is labeled with a detectable chemical group, preferably an enzymatically active group such as an enzyme, a fluorescer, a chromophore, a luminescer, a specifically bindable ligand, or a radioisotope.

8. The method of Claim 7 wherein the labeled antibody reagent which becomes bound to said duplexes is separated from that which does not become so bound and wherein the detectable chemical group is measured in one of the separated fractions.

9. The method of any of Claims 1 to 8 wherein the particular polynucleotide sequence to be determined is RNA or DNA and said probe is substantially composed of RNA.

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10. The method of any of Claims 1 to 8 wherein the particular polynucleotide sequence to be determined is RNA and said probe is substantially composed of DNA.

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EUROPEAN SEARCH REPORT

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl 4)
D,P X	EP-A-0 163 220 (MILES LABORATORIES INC.) * Whole document *	1-10	C 12 Q 1/68 G 01 N 33/543 G 01 N 33/569
X	EP-A-0 144 913 (MILES LABORATORIES INC.) * Whole document *	1-10	
X	EP-A-0 133 671 (MILES LABORATORIES INC.) * Abstract; pages 21-26; claims 1,3,4,10 *	1-10	
X	EP-A-0 135 159 (CETUS CORP.) * Whole document *	1-10	
A	EP-A-0 145 356 (NATIONAL RESEARCH DEVELOPMENT) * Abstract; claims 1-9; figure 1/1 *	1,6	**TECHNICAL FIELDS SEARCHED (Int Cl 4)** C 12 Q
A	EP-A-0 079 139 (ORION CORP. LTD.) * Abstract; claims 1-12 *	1,6	
A	EP-A-0 133 288 (MILES LABORATORIES INC.) * Pages 3,4,12-15; examples 1-3; claims 1-10 *	1-5	
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The present search report has been drawn up for all claims

Place of search THE HAGUE	Date of completion of the search 24-10-1986	Examiner OSBORNE H.H.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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D : document cited in the application
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	DOCUMENTS CONSIDERED TO BE RELEVANT		Page 2
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	GENE, vol. 21, 1983, pages 77-85, Elsevier Biomedical Press; M. RANKI et al.: "Sandwich hybridization as a convenient method for the detection of nucleic acids in crude samples" * Whole document *	1,6	
D,A	US-A-4 483 920 (D. GILLESPIE et al.)		

The present search report has been drawn up for all claims

TECHNICAL FIELDS
SEARCHED (Int. Cl.4)

Place of search	Date of completion of the search	Examiner
THE HAGUE	24-10-1986	OSBORNE H.H.

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